IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

in re patent application of:
Roland Reiner et al.
Appl. No. 10/599.980
For: **Injectable Crosslinked And Uncrosslinked Alginates And The Use Thereof In Medicine And In Cosmetic Surgery**

Confirmation No.:
Art Unit: 1623
Examiner: KRISHNAN, GANAPATHY

Inventor's Declaration

1. **Inventors of the instant application**

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2. **Examples to which the inventors refer in forming their declaration:**

Example 3 of the disclosure of the above defined US patent application:

"Preparation of barium-crosslinked microcapsules of alginate

The alginate solution (see Example 1) is first subjected to sterile filtration under laminar flow. This is carried out by filtration through a 0.2 µm sterile filter. The solution is forced slowly through the filter and collected in a sterile 50 ml centrifuge tube. The tube is closed and labelled. The alginate solution is centrifuged at 1,000 ± 100 rpm for 5 ± 1 min at room temperature. The autoclaved encapsulating apparatus is screwed with 2 screws at a height of 10 ± 2 cm to an electrically regulatable syringe advancer. A compressed air hose is connected to the apparatus with a clamp and the compressed air is then regulated to a value of 3 ± 1.0 l/min. An open Petri dish is placed under the drop formation nozzle. Three portions of 1 ± 0.1 ml each of sterile water are flushed through the nozzle channel with a 1 ml syringe for cleaning. The speed of the advancers is adjusted to 3 ± 0.5 units. A sterile 1 ml syringe is filled with the centrifuged alginate solution without air bubbles and fitted on to the nozzle. In order to prepare capsules having a diameter of 200 µm, the diameter of the channel of the drop formation nozzle must be approx. 100 µm. The nozzle advancer is switched on and a new Petri dish with 40 ml of precipitating bath (see Example 2) is placed under the nozzle. Drops of alginate now form at the end of the nozzle which are torn off by the stream of air and fall into the precipitating bath. The encapsulation is carried out until the syringe is empty. The capsules cure in the precipitating bath for 10 minutes. When the alginate capsules have

cured, they are transferred into a 50 ml centrifuge tube with 10 ± 1 ml of 0.9 % NaCl solution. The capsules are then washed 5 times with 10 ± 0.1 ml of 0.9 % NaCl. Thereafter, the capsules are transferred into 10 ml of 6 mM Na_2SO_4 solution and placed in an incubator (37°C, 5 % CO_2) for 20 ± 2 min. Thereafter, the Na_2SO_4 solution is stripped off and 10 ± 0.1 ml of 0.9 % NaCl solution are added and incubation is carried out in an incubator (37°C, 5 % CO_2) for 20 ± 2 min. Thereafter, the capsules are washed 5x with 10 ± 1 ml of 0.9 % NaCl solution. The capsules are then transferred into a polystyrene tube with physiological saline solution and transferred with a high packing density into disposable injection syringes under sterile conditions in units of 1 ml each and marketed."

3. **Conclusions and reasoning, which supports the non-obviousness of pending claims**

In the interview conducted on 11 April 2011 in the above referenced patent application with the Examiner Ganapathy Krishnan and the Supervisory Shaojia Jiang, the Examiner requested evidence, that the microparticles of the present invention, e.g. as prepared via Example 3 as shown above, are in fact fully cross-linked.

The full cross-linking is given by an excess of divalent counter ions like barium in the cross-linking solution which will diffuse rapidly into the alginate beads.

Evidence for the full cross-linking is given by the following calculation.

4. **Calculation of potential binding sites and available divalent cations:**

There is no physical or spectroscopic method available to experimentally determine full or partial cross-linking in alginate beads. However, full crosslinking may be determined indirectly by measurement of the contents of the bead's components and the following calculation shown below:

Alginate microparticles of the invention have been analysed by the Applicant in terms of their contents:

One microparticle consists of ~10mg/ml alginate ~300µg/ml barium (2.2 µmol) and ~500 µg/ml calcium (12 µmol) (Alginate: C6H8O6; MW of the monomers = 176 g/mol). 10mg alginate corresponds to 57 µmol monomers. Analysis has also shown that the alginate of the microparticle consists out of 40% of the monomers guluronic acid which corresponds to 23 µmol guluronic acid (40% of 57 µmol). The remainder corresponds to mannuronic acid residues which are not involved in the cross-linking.

From e.g. Draget et al (2005) or other publications it is known that for the cross linking process (resulting in the formation of a so called "egg box", for further details see p. 13, Fig. 7 of Draget et al., which is attached as Appendix A) 4 guluronic acid residues (2 of each chain) are cross-linked by one divalent cation, be it barium or calcium, whereby barium ions have a

significantly stronger binding affinity than calcium ions to guluronic acid residues. As 4 guluronic acid residues are linked by one divalent barium cation, 23µmol guluronic acid (as contained in the inventive bead, see above) need about 6 µmol divalent cations for cross-linking. All barium ions in the inventive bead (2.2 µmol) are involved in the formation of egg box structures due to their strong binding affinity. That results in > 30% (2.2/6) of all guluronic acid residues being cross linked in "egg box structures" by barium ions in the inventive microparticle.

However, guluronic acid residues are exclusively accessible for the formation of egg box structures, if they are provided in homopolymeric regions (of at least 10 contiguous guluronic acid residues) in the alginate that form a tight guluronic acid – barium egg box formation (see Fi. 7 of Draget et al.). Guluronic acid residues, which are involved of blocks of smaller size (heteropolymeric regions) in the alginate, cannot form egg boxes (for structural reasons). These guluronic acid residues located in heteropolymeric alginate regions show affinity to calcium ions which link two guluronic acid residues (each of a distinct chain), thereby contributing to full crosslinking of alginate.

The above measurement allows to conclude that all of the glucuronic acid residues which are not arranged in larger homopolymeric regions and are, therefore, not involved in egg box formation (app. 65%), are actually cross-linked by the excess amount of calcium ions determined for the inventive bead. That is confirmed by the following calculation: One inventive bead contains (as determined by the Applicant's analysis) 12µmol calcium. One bead contains app. 14 µmol of guluronic acid residues not bound by barium (app. 4 x 2.2 µmol guluronic acid residues form egg boxes, see above, which results in 23 µmol (total amount) minus 9 µmol guluronic acid residues). That remainder of 14 µmol guluronic acid residues may be bound by an excess of 12 µmol calcium ions, since each calcium ion allows to cross link 2 guluronic acid residues (herein, a maximum of 24 µmol guluronic acid residues).

Accordingly, the excess amount of calcium ions in one inventive bead ensures full cross-linking of all the remaining heteropolymeric guluronic acid residues (not forming egg boxes) which are available for calcium cross linking.

Thus, the microparticles according to the present invention are in fact fully cross-linked.

I declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

In knowledge of the above, I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true.

03.06.2011
date

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10.1.2

Selective Ion Binding

The basis for the gelling properties of alginates is their specific ion-binding characteristics (Haug, 1964; Smidsrød and Haug, 1968b; Haug and Smidsrød, 1970; Smidsrød, 1973, 1974). Experiments involving equilibrium dialysis of alginate have shown that the selective binding of certain alkaline earth metals ions (e.g., strong and cooperative binding of Ca^{2+} relative to Mg^{2+}) increased markedly with increasing content of α-L-guluronate residues in the chains. Poly-mannuronate blocks and alternating blocks were almost without selectivity. This is illustrated in Figures 5 and 6, where a marked hysteresis in the binding of Ca^{2+} ions to G-blocks also is seen.

The high selectivity between similar ions such as those from the alkaline earth metals indicates that some chelation caused by structural features in the G-blocks takes place. Attempts were made to explain this phenomenon by the so-called "egg-box" model (Grant et al., 1973), based upon the linkage conformations of the guluronate residues (see Figure 1b). NMR studies (Kvam et al., 1986) of lanthanide complexes of related compounds suggested a possible binding site for Ca^{2+} ions in a single alginate chain, as given in Figure 7 (Kvam 1987).

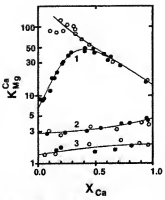

Fig. 6 Selectivity coefficients, K^{Ca}_{Mg} as a function of ionic composition (X_{Ca}) for different alginate fragments. Curve 1: Fragments with 90% guluronate residues. Curve 2: Alternating fragment with 38% guluronate residues. Curve 3: Fragment with 90% mannuronate residues. •: Dialysis of the fragments in their Na^+ form. ○: Dialysis first against 0.2 M $CaCl_2$, then against mixtures of $CaCl_2$ and $MgCl_2$

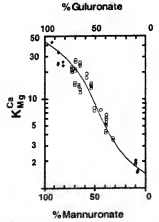

Fig. 5 Selectivity coefficients, K^{Ca}_{Mg}, for alginates and alginate fragments as a function of monomer composition. The experimental points are obtained at $X_{Ca} = X_{Mg} = 0.5$. The curve is calculated using $K^{Ca}_{Mg\ guluronate} = 40$ and $K^{Ca}_{Mg\ mannuronate} = 1.8$

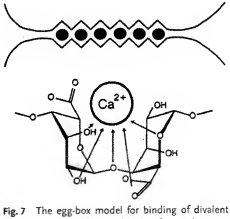

Fig. 7 The egg-box model for binding of divalent cations to homopolymeric blocks of α-L-guluronate residues, and a probably binding site in a GG-sequence.